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High-performance liquid chromatography-based assay for glutathione transferase theta 2 activity: Application to characterize interindividual variability in human liver fractions

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ABSTRACT

Human glutathione transferase T2-2 (GSTT2-2) is one of the enzymes considered to play a role in inactivation of toxicants and carcinogens. The expression level of this enzyme is determined by genetic and environmental factors, which may lead to differences in susceptibility. As a specific assay for GSTT2-2 so far a spectroscopical assay based on GSH-conjugation of menaphthyl sulfate (MSu) was used. This spectrophotometric assay, however, appeared too insensitive to accurately quantify the GSTT2-2 activities in a panel of 20 human liver samples. More recently, expression levels of GSTT2-2 in biological samples are quantified by measuring mRNA levels. Since mRNA-levels do not always correlate well with enzyme activity, a specific and sensitive assay is required. In the present study a highly sensitive high-performance liquid chromatography (HPLC)-based method was developed. By applying the new method, firstly, the specificity of GSTT2-2 among 15 recombinant human GST isoforms in catalyzing GSH-conjugation of MSu was confirmed. In addition, a 65-fold inter-individual variation of GSTT2-2 activity was found from the individual liver fractions. By applying the method to individual liver fractions, a 65-fold inter-individual variation of GSTT2-2 activity was found. As a second application, the role of GSTT2-2 in GSH-conjugation of the environmental carcinogen 1-methylpyrene sulfate (MPS) was studied by correlation analysis with GSTT2-2-catalyzed MSu conjugation. The relatively poor correlation suggested that other GSTs also contribute to MPS-conjugation, as confirmed by incubations with recombinant GSTs.

1. Introduction

Human glutathione transferases (GSTs) are important phase II enzymes ubiquitously expressed in different tissues. Because they catalyze the inactivation of electrophiles and hydroperoxides using the cofactor glutathione (GSH), they play an important protective role in the elimination of potentially toxic and carcinogenic chemicals, including drugs, pesticides, and environmental pollutants. Based on their subcellular localizations, human GSTs can be divided into three subclasses namely cytosolic GSTs, microsomal GSTs and mitochondrial GSTs [1]. The cytosolic human GSTs are dimeric proteins and are the most extensively-studied subclass regarding their contributions as a protective enzyme system. The human cytosolic GSTs are further divided in seven distinct classes: Alpha (A), Mu (M), Pi (P), Theta (T), Omega (O), Sigma (S), and Zeta (Z). Amongst them, the GST theta class enzymes are relatively less studied compared to the major cytosolic GSTs. However, the genetic polymorphisms of Theta class GSTs have been associated with increased risk for chemical-induced DNA-damage which my lead to increased risk of cancers [2].

The human Theta class GST is composed of two isoforms, GSTTT1-1 and GSTT2-2. These GSTs differ from the other cytosolic enzymes by having a relatively low affinity to GSH, which explains

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; GSH, glutathione; GST, glutathione transferase; $HClO_4$, perchloric acid; HLC, human liver cytosol; HPLC, high-performance liquid chromatography; IDRs, idiosyncratic drug reactions; MP, 1-methylpyrene; MPdA, N^6-(1-methylpyrenyl)-2′-deoxyadenosine; MPdG, N^2-(1-methylpyrenyl)-2′-deoxyguanosine; MPMA, S-((pyrene-1-yl) methyl) N-acetylcysteine; MPS, 1-methylpyrene sulfate; MP-SG, 1-methylpyrene GSH conjugate; MSG, 1-menaphthyl sulfate GSH conjugate; MSu, 1-menaphthyl sulfate; PAHs, polycyclic aromatic hydrocarbons.

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that they cannot be isolated from tissue extracts by GSH affinity chromatography [3]. Furthermore, GSTT1-1 and GSTT2-2 do not conjugate 1-chloro-2,4-dinitrobenzene (CDNB), which is a general substrate for all other GSTs [4]. Substrates for the theta class GSTs are 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) which is selective for GSTT1-1 and 1-menaphthyl sulfate (MSu) which appeared to be a substrate for GSTT2-2 [4,5]. However, the selectivity of GSH-conjugation of MSu for GSTT2-2 has not been studied with the full spectrum of human GSTs yet. So far, the GSH-conjugation of MSu has been performed by a spectrophotometric method [6]. Although this method has advantages of low-cost lab facilities and quick assay duration, disadvantages are the lacking of sensitivity and accuracy with complicated matrices. These disadvantages limit its application to samples with low GSTT2-2 expression levels, such as cell lines. As alternative methods to determine GSTT2-2 expression levels, mRNA levels and western blot assays are used [7,8]. However, it has been demonstrated that mRNA-levels of drug metabolizing enzymes sometimes poorly correlate with protein levels and enzyme activity [9,10]. Furthermore, western blot analysis is not able to distinguish between active and inactive enzymes. Therefore, a more sensitive method for measuring of GSTT2-2-catalyzed GSH-conjugation of MSu is required.

Although it has been demonstrated by immunochemical methods that human GSTT2-2 is expressed in multiple organs, the expression level and variability of GSTT2-2 activity in human tissues is still poorly studied. Inter-individual variability of GSTs is generally considered to have a significant impact on internal exposure to reactive drug metabolites, which may thereby affect susceptibility to toxicity and carcinogenicity [1]. Although in contrast to GSTT1-1, no null genotype has been identified for *GSTT2*, a deletion of the neighboring *GSTT2b* pseudogene was shown to result in a very strong decrease in the enzyme expression level [11]. This deletion has been associated with an increased risk of esophageal squamous cell carcinoma [12]. Furthermore, a G537A mutation in the promotor area of *GSTT2*, which leads to lower transcription of *GSTT2*, has been associated with an increased risk for colorectal cancer [13].

Mutagens that have been identified as substrates for GSTT2-2 include N-acetoxy-PhIP, secondary lipid peroxidation products, organic hydroperoxides, and sulfate esters of methylated polycyclic aromatic hydrocarbons (PAHs) [14]. The selectivity towards sulfate esters can be rationalized by the presence of a sulfate binding pocket which was found in the crystal structure of GSTT2-2 [15]. 1-Methylpyrene (MP) is one of the methylated polycyclic aromatic hydrocarbons and has been detected in cigarette smoke at levels exceeding that of benzo[α]pyrene [16,17]. As the ultimate carcinogen of MP, 1-methylpyrene sulfate (MPS) is formed by sequential benzylic hydroxylation and sulfation by catalysis of sulfotransferases. MPS has been shown to bind covalently to DNA forming N^2-(1-methylpyrenyl)-2′-deoxyguanosine (MPdG) and N^6-(1-methylpyrenyl)-2′-deoxyadenosine (MPdA) in *in vitro* and *in vivo* experiments [18]. The excretion of S-((pyrene-1-yl) methyl) N-acetylcysteine (MPMA) in rat urine [19] indicated an involvement of GSH-conjugation pathway *in vivo*. Although several non-reactive sulfate esters of methylated PAHs have been tested with GSTT2-2, the role of human GSTs in detoxifying MPS has not been characterized yet.

The major aims of the present study were, i) to establish a sensitive HPLC-UV-based method to enable accurate quantification of human GST T2-2 activity using MSu as a substrate; ii) to apply this method for the characterization of the specificity in catalyzing MSu GSH-conjugation by comparing 15 recombinant human GSTs; iii) to determine the inter-individual variation of enzyme activity and to estimate the expression level of GSTT2-2 in a panel of human liver cytosol (HLC) from 20 different donors; iv) to characterize the role of GSTT2-2 in the detoxification of MPS by applying correlation analysis and recombinant human GSTs.

2. Materials and methods

2.1. Materials

Recombinant human GST isoforms A1-1, A2-2, A3-3, A4-4, M1-1, M2-2, M3-3, M4-4, P1-1*A, P1-1*B, P1-1*C, P1-1*D, K1-1, T1-1, and T2-2 were expressed and purified according to the protocols published previously [20]. Pooled HLC was purchased from BD Biosciences (Breda, The Netherlands). Individual HLC was prepared from liver fractions from 20 donors kindly provided by Kaly-Cell (Strassbourg, France). MSu and S-(1-menaphthyl) glutathione (MSG) were synthesized according to previously described methods [21,22]. MPS was kindly provided by Prof. Hansruedi Glatt (German Institute of Human Nutrition, Berlin, Germany). GSH, ammonium acetate, formic acid and ammonium hydroxide were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). All other chemicals and reagents were of analytical grade and obtained from standard suppliers.

2.2. Analysis of GSH-conjugation of MSu by spectrophotometry and by HPLC-UV

The GSH-conjugation of MSu was determined by the spectrophotometric method as described by [8]. Incubations were performed in a final volume of 1 mL 100 mM Kpi buffer pH 7.4 (prepared by mixing 100 mM KH_2PO_4 and 100 mM KH_2PO_4 at ratio of 1.98:8.02) containing recombinant human GSTs or HLC (pooled and individual), 5 mM GSH and 100 μM Msu and at an incubation temperature of 37 °C. After starting the reaction by addition of GSH, the increase in absorbance at 298 nm was recorded for 10 min using an Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech, Cambridge, England).

The purposed HPLC-UV method, more accurate and sensitive than the spectrophotometric one, was developed to quantify the enzyme activity in incubations with HLC. To this end, incubations as described above were terminated by the addition of 1% perchloric acid ($HClO_4$) (final concentration) and centrifuged at 14,000 rpm to precipitate the proteins. The supernatants were analyzed on a Shimadzu HPLC system consisting of two LC-20AD binary pumps, a SIL-20AC auto-sampler (cooled at 4 °C), and a SPD-20A UV/VIS detector set at 298 nm. Chromatographic separation of analytes was performed with a Luna 5 μm C8 column (50 mm × 3 mm) and a gradient composed of solvent A (10 mM ammonium acetate, pH 8.0 adjusted with ammonium hydroxide) and solvent B (100% acetronitrile). The flow rate was set at 0.5 mL/min and the gradient was programmed as follows: 0–2 min, isocratic 10% B; 2–12.5 min, linear increase from 10% B to 35%B, 12.5–13 min, linear increase from 35% B to 99% B; 13–13.5 min, linear decrease from 99% B to 10% B; 13.5–20 min isocratic at 10% B.

2.3. Isoenzyme selectivity of GST-catalyzed GSH conjugation of MSu

Previous studies on the specificity of GSH-conjugation by GSTT2-2 were performed with an incomplete set of human GSTs. Therefore, in the present study 15 recombinant human GSTs were incubated at a GST concentration of 50 nM with 100 μM MSu at 37 °C for 10 min. The major hepatic GST isoforms, GSTA1-1, A2-2, and M1-1 were also incubated at an enzyme concentration of 100 μM, which is the highest concentration found in HLC [23,24]. All incubations were performed in 100 mM potassium phosphate (KPi) buffer, pH 7.4, in the presence of 5 mM GSH at a final volume of 200 μL in duplicate. Incubations were initiated by the addition

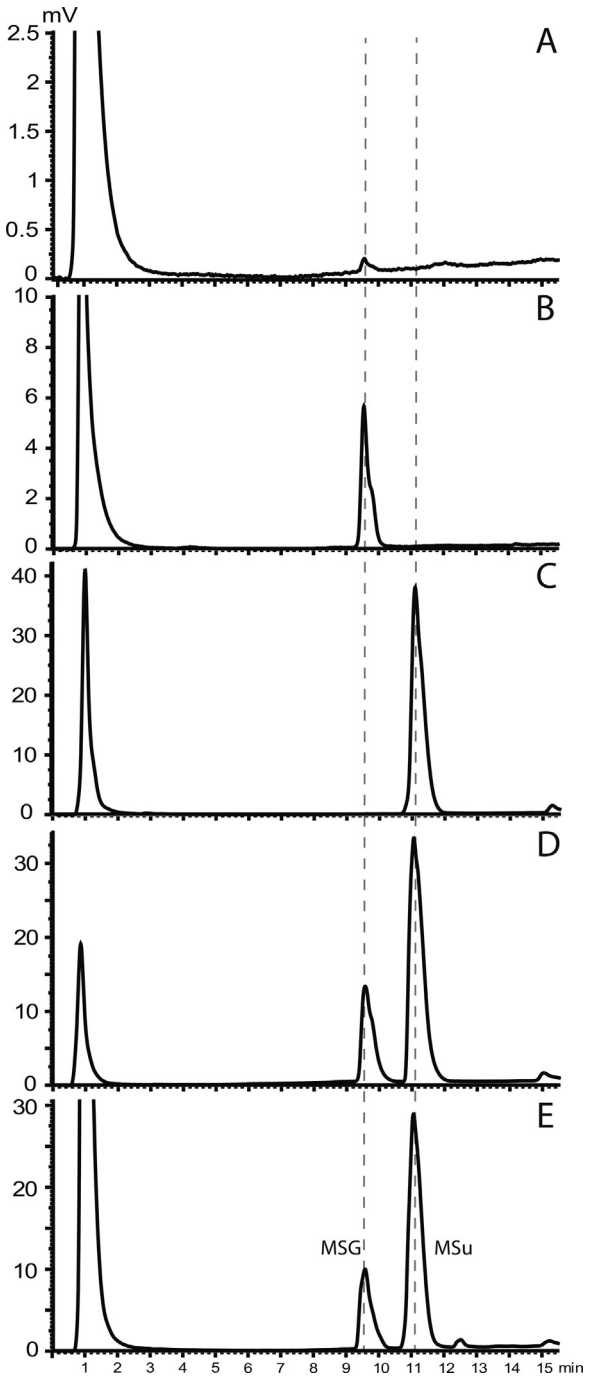

Fig. 1. Representative chromatograms of MSG and MSu from standard and incubation samples, analyzed by HPLC-UV-based assay established in the current study. (A) MSG reference sample at 0.13 μM, lowest limit of quantification; (B) MSG reference sample at 6.5 μM; (C) incubation sample containing only 100 μM MSu and 5 mM GSH; (D) incubation sample of 100 μM MSu with 0.2 μM recombinant human GSTT2-2 in the presence of 5 mM GSH; (E) incubation sample of 100 μM MSu with 5% of human liver cytosol from donor S1399T in the presence of 5 mM GSH.

of MSu and terminated with ice-cold $HClO_4$ at a final concentration of 1% (*v*/*v*) and cooled on ice for 10 min. Precipitates were removed by centrifugation for 15 min at 14,000 rpm. The supernatants were analyzed by HPLC-UV as described in Section 2.2.

2.4. Enzyme kinetics of GSH conjugation of MSu catalyzed by recombinant human GSTT2-2 and pooled HLC

The enzyme kinetical parameters of GSH conjugation of MSu by recombinant hGSTT2-2 were determined by varying the MSu-

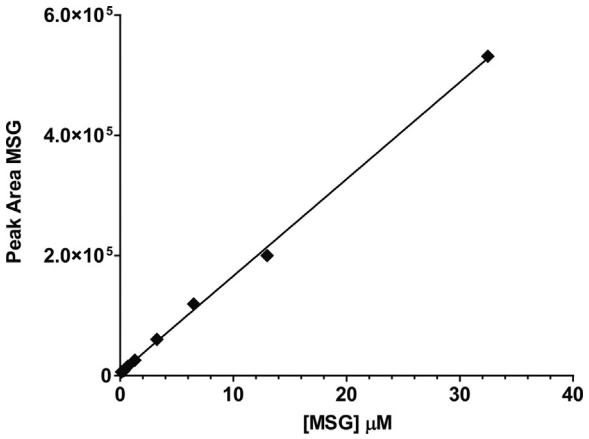

Fig. 2. Calibration curve of MSG ranging from 0.13 to 32.5 μM.

concentration from 0.5 to 50 μM. Incubations were performed at 37 °C in 100 mM KPi buffer, pH 7.4, in the presence of 5 mM GSH and 50 nM recombinant human GSTT2-2. This concentration of GSTT2-2 was selected based on the linear range of enzyme concentration-dependency of MSG formation (Supplemental Fig. S1). All incubations were performed in a total volume of 200 μL and were pre-incubated at 37 °C for 5 min. Reactions were started by the addition of GSH. After 2 min, the incubations were terminated by addition of $HClO_4$ (1% final concentration, *v*/*v*). The analysis of samples was conducted by the HPLC-UV method as described in Section 2.2. Enzyme kinetics of GSH conjugation of MSu with pooled HLC were performed using 0.72 mg/mL HLC. MSu concentrations ranging from 0.1 to 50 μM were used to study the enzyme kinetics. Incubations were performed at 37 °C for 10 min based on linearity of reaction (Supplemental Fig. S2). All other conditions were the same as described for recombinant human GSTT2-2.

Enzyme kinetical parameters were calculated by nonlinear regression using the Michaelis–Menten equation or the substrate inhibition equation with GraphPad Prism 5 software (San Diego, CA, USA).

2.5. Characterization of GSTs involved in the GSH-conjugation of MPS

To investigate which GSTs are able to catalyze GSH-conjugation of MPS, MPS was incubated at a concentration of 100 μM with the 15 available recombinant human GST isoforms at an enzyme concentration of 8 μM. Incubations were performed in 100 mM KPi buffer, pH 7.4, in the presence of 5 mM GSH for 1 h at 37 °C. Reactions were started by adding MPS and terminated by adding an equal volume of ice-cold acetonitrile. Samples were cooled on ice for 10 min and centrifuged for 15 min at 14000 rpm. The GSH-conjugate formed, MP-SG, was quantified using the HPLC method of Reinen et al. [25], with minor modifications. Samples at 50 μL of supernatant were analyzed using a Shimadzu HPLC system consisting of two LC-20AD binary pumps, a SIL-20AC auto-sampler, and a SPD-20A UV/VIS detector coupled with a reversed-phase C18 column (ChromSpher, 5 μm, 100 × 3 mm, Chrompack, Middelburg, The Netherlands). A gradient composed of solvent A (5% of acetonitrile and 95% of 10 mM ammonium acetate, pH adjusted to 5 with formic acid) and solvent B (95% of acetonitrile and 5% of 10 mM ammonium acetate) was used. The gradient was programmed as follows: gradient elution from 5% B to 90% B for 4.5 min, isocratic elution at 90% B for 4 min, gradient elution back to 5% B from 8.5 to 9 min, and isocratic elution at 5% B from 9 to 15 min. The flow rate was 0.5 mL/min and UV detection was set at a wavelength of 346 nm. MP-SG reference was obtained by quantitative conversion

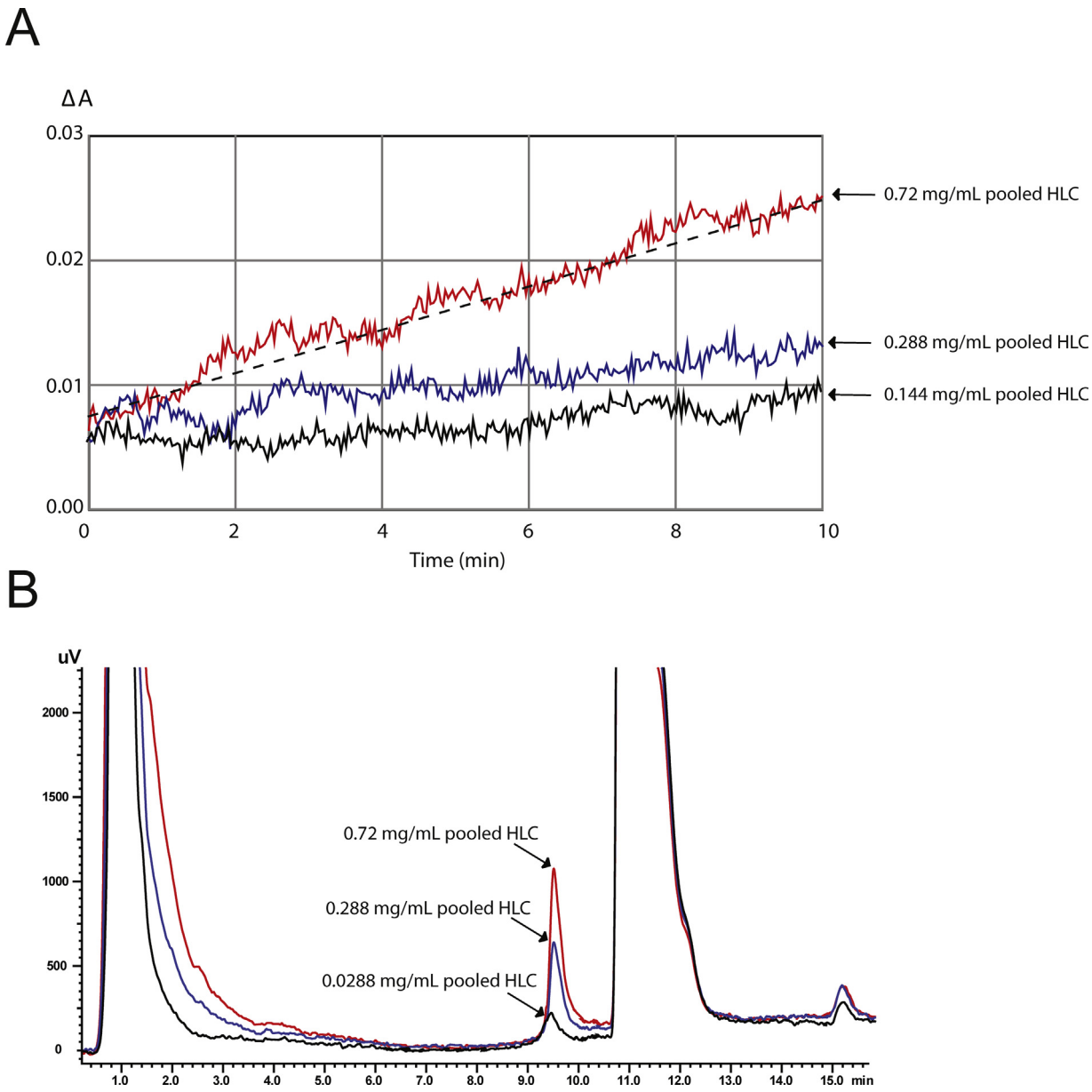

Fig. 3. Comparison of HPLC–based and spectrophotometric assays for GSTT2-2 activity measurement in pooled human liver cytosol (HLC) using MSu as a substrate. (A) UV spectra of absorbance at 298 nm of incubations containing 0.144 (black), 0.288 (blue), and 0.72 (red) mg/mL pooled HLC; (B) HPLC chromatograms of MSG formation from incubations containing 0.0288 (black), 0.288 (blue), and 0.72 (red) mg/mL pooled HLC. Details of incubation conditions was described in the Materials and Methods section. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of samples with varying concentrations of MPS, since MP-SG is the only metabolite in this biotransformation reaction. The standard curve of MP-SG appeared linear within the range of 0.26–52.63 μM.

The correlation between GST-conjugation of MPS and MSu was studied by using a panel of HLC from 20 donors. Each individual HLC fraction was incubated for 30 min at 37 °C with 100 μM MSu or MPS, at a protein concentration of 2 mg/mL and in presence of 5 mM GSH. Reactions were performed in a final volume of 200 μL and in duplicate and started and terminated as described above. Correlation analysis of formation rates between MP-SG and MSG was performed using a Spearman's correlation test with GraphPad Prism 5 software (San Diego, CA, USA). $P < 0.05$ was regarded as statistically significant and all tests were two-sided.

3. Results

3.1. Validation of the HPLC-UV method for the analysis of MSG and MSu

A sensitive HPLC-UV-based assay for the quantification of MSG formed by GSTT2-2 was established. Representative HPLC chromatograms of reference compounds and incubation samples are presented in Fig. 1. The retention times of MSG and MSu were 9.6 and 11.1 min, respectively. When incubating MSu with both recombinant human GSTT2-2 (Fig. 1D) and HLC (Fig. 1E), MSG was found to be the only metabolite. No MSG formation was found in incubations in the absence of GSTT2-2 fraction (Fig. 1C). The lowest limit of quantification of MSG using this HPLC-UV method was 0.13 μM,

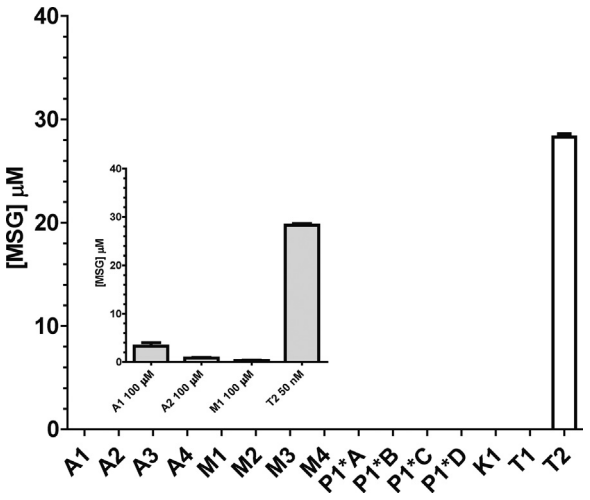

Fig. 4. Specificity of GSTT2-2-catalyzed menapthyl GSH-conjugate (MSG) formation. Incubation conditions, each recombinant human GST isoform at 50 nM was incubated with 100 μM of menapthyl sulfate (MSu) in the presence of 5 mM GSH for 10 min at 37 °C. Insert show the incubations of recombinant human GSTA1-1, GSTA2-2, GSTM1-1 at 100 μM, and GSTT2-2 at 50 nM incubating with 100 μM MSu in the presence of 5 mM GSH for 10 min at 37 °C. Each bar represents average ± range from two separate incubations.

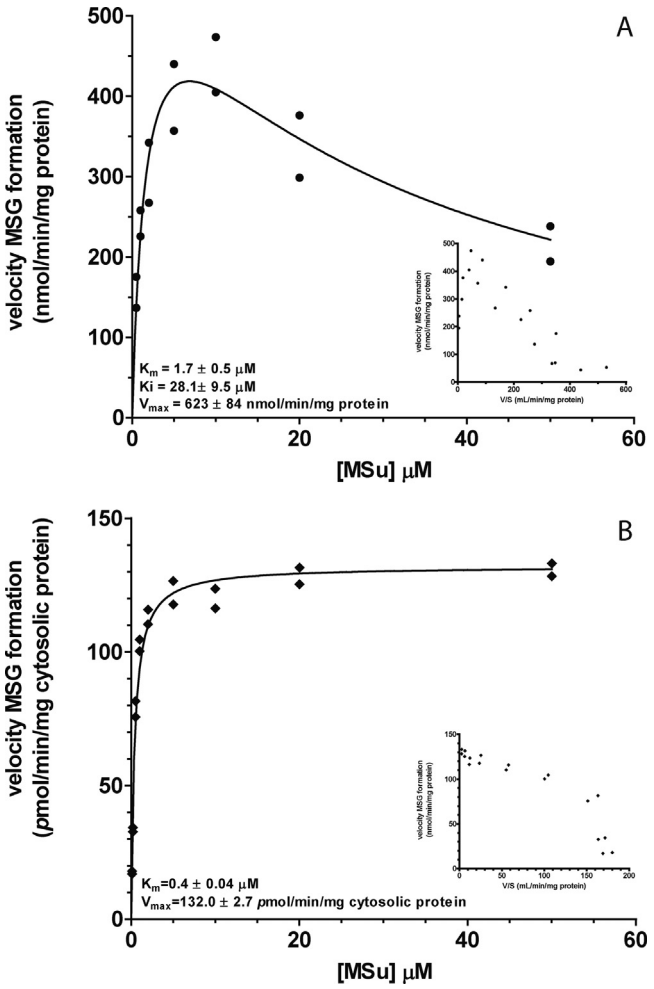

Fig. 5. Enzyme kinetics of human recombinant GSTT2-2 (A) and pooled HLC (B) probed by MSu GSH-conjugation reaction. Incubation conditions, (A) recombinant human GSTT2-2 at 50 nM was incubated with MSu ranging from 0.5 to 50 μM in the presence of 5 mM GSH at 37 °C for 2 min; (B) pooled HLC at 0.72 mg/mL was incubated with MSu ranging from 0.1 to 50 μM in the presence of 5 mM GSH at 37 °C for 10 min. Solid lines are obtained by nonlinear regression using the Michaelis–Menten equation or substrate inhibition equation. Inserts show corresponding Eadie-Hofstee plot.

as shown in Fig. 1A. Linear calibration curve of MSG were obtained from 0.13 to 32.5 μM, as shown in Fig. 2.

To compare the sensitivity of the HPLC-UV method with that of the spectrophotometric assay of Gillham [8], incubations were performed at different concentrations of pooled HLC. As shown in Fig. 3A, at a HLC-concentration of 0.288 mg/mL the spectrophotometric assay showed only a weak slope with an increase in absorbance over 10 min of approximately 3 times to the noise. Only at a HLC-concentration of 0.72 mg/mL the increase in absorbance was more than 10 times to the noise, which is usually considered as the lowest limit of quantification. However, when analyzed by HPLC-UV the MSG could still be quantified accurately in incubation containing 0.0288 mg/mL pooled HLC, Fig. 3B. Thus the sensitivity of the current HPLC-UV assay is estimated to be approximately 25-fold higher than that of the spectrophotometric assay.

3.2. Isoenzyme selectivity of GST-catalyzed GSH conjugation of MSu

To confirm the selectivity of MSu as a GSTT2-2-specific substrate, 15 recombinant human GST isoforms were incubated at an enzyme concentration of 50 nM with 100 μM MSu and in the presence of 5 mM GSH. As shown in Fig. 4, only GSTT2-2 exhibited a high activity in the formation of MSG. None of the other GST isoforms showed significant MSG-formation when incubated at 50 nM enzyme concentration. Because GSTA1-1, GSTA2-2, and GSTM1-1 can have hepatic concentrations up to 100 μM, these recombinant enzymes were also incubated at 100 μM with MSu at identical condition as mentioned above. The insert in Fig. 4 shows that these GSTs at 100 μM only formed small amounts of MSG, being less than 10% of the amount formed by 50 nM GSTT2-2.

3.3. Enzyme kinetics of GSH-conjugation of MSu by recombinant human GSTT2-2 and pooled HLC

To further characterize the GSH-conjugation of MSu by recombinant human GSTT2-2 and pooled HLC, enzyme kinetic studies were performed. Protein concentrations and incubation times were first optimized with regard to linearity of product formation, Supplemental Fig. S2. As shown in Fig. 5A, for recombinant human GSTT2-2, the formation of MSG increased up to an MSu concentration of 10 μM. When MSu concentrations were higher than 10 μM, a decrease in MSG formation was observed. By fitting data into substrate inhibition equation, K_m was calculated as 1.7 ± 0.5 μM, V_{max} as 623 ± 84 nmol/min/mg protein, and K_i of 28.1 ± 9.5 μM. Interestingly, no substrate inhibition pattern was observed for pooled HLC kinetics (Fig. 5B). The K_m value in this case was 0.4 ± 0.04 μM, and V_{max} value was 132.0 ± 2.7 pmol/min/mg cytosolic protein.

3.4. Characterization of GSTs involved in the GSH-conjugation of MPS

To characterize the GSTs involved in the GSH-conjugation of MPS, 105 μM MPS was incubated with 8 μM recombinant human GST isoforms for 5 min in the presence of 5 mM GSH. As in case of MSu, under these conditions no spontaneous GSH-conjugation was detected. As shown in Fig. 6A, GSTT2-2 was the most active isoform. A few GST isoforms, such as GSTA3-3, A4-4, P1-1*B, P1-1*C and P1-1*D, weakly catalyzed the GSH-conjugation of MPS, with converting less than 5% MPS to MP-SG. In contrast, GSTT2-2 exhibited a remarkably high activity in catalyzing this reaction, with a formation of 45 μM MP-SG. By normalizing GSTT2-2 activity in catalyzing

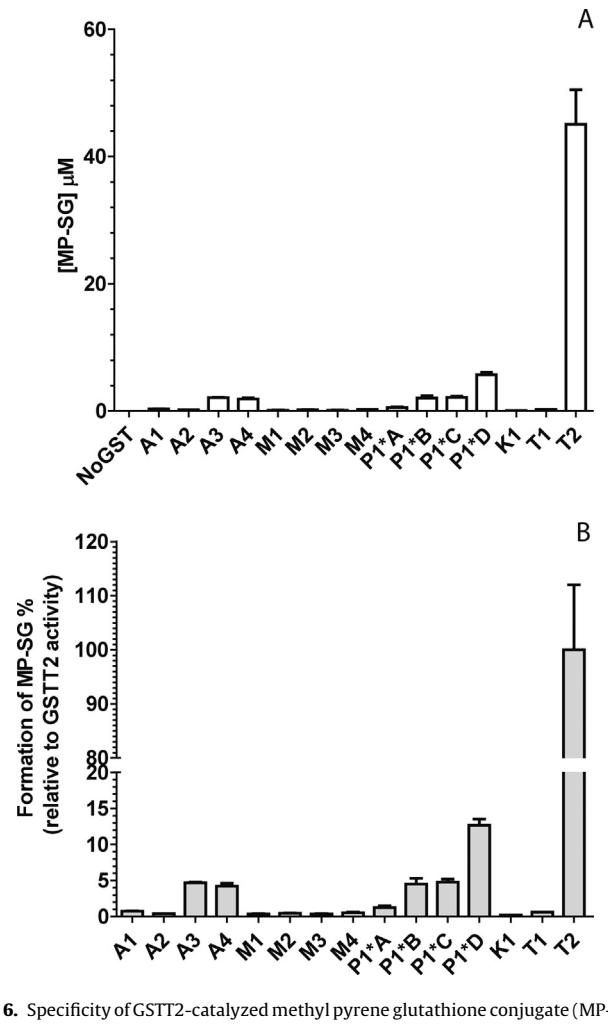

Fig. 6. Specificity of GSTT2-catalyzed methyl pyrene glutathione conjugate (MP-SG) formation. Each GST isoforms at 8 μM was incubated with 105 μM of methyl pyrene sulfate (MPS) in the presence of 5 mM GSH for 5 min at 37 °C. (A) concentrations of MP-SG formation; (B) percentage of MP-SG formation with GSTT2-2-catalyzed formation of MP-SG normalized as 100%. Data are presented as average ± range of two separate incubations.

the formation of MP-SG as 100%, all other GST isoforms showed activities not higher than 13% (Fig. 6B). Table S1 shows the values of formation of MP-SG by each GST isoform.

3.5. Correlation analysis of activities of GSH-conjugation of MSu and MPS in 20 HLC donors

To investigate the correlation between GSH-conjugation of MPS and human GSTT2-2 activity, MP-SG and MSG formation were measured using a panel of HLC-fractions from 20 donors. As shown in Fig. 7A, a 65-fold variability was observed using MSu as a substrate (between S1329T and S1399T), reflecting a large variability in hepatic GSTT2-2 expression. When using MPS as a substrate, however, only a 3.8-fold difference was found between the most and least active HPLC-fractions, S1449T and S1343T, respectively. Table S2 showed the detailed values of activity of each liver donor in catalyzing formation of MP-SG and MSG. These results implied the possibility of large variations of both expression level of GSTT2-2 and detoxifying profiles of MPS among population.

Although, a significant correlation was found between GSH-conjugation of MPS and MSu, Fig. 7B (Spearman $r = 0.86$, $p < 0.0001$) the line does not cross the origin, suggesting the contribution of other cytosolic GSTs, consistent with the results with the recombinant GSTs, Fig. 6.

4. Discussion

In the current study, an HPLC-UV method was established for the accurate quantification of human GSTT2-2 in biological samples. With this method MSG concentration low to 0.13 μM can be accurately quantified. Although several previous studies have used the spectrophotometric method to determine GSTT2-2 activity [4,6], the HPLC-UV method was found to be more than 25 times more sensitive (Fig. 3). The more sensitive HPLC-based method can be used as a reliable tool for the analysis of in vitro samples containing low GSTT2-2 activity, of which the sensitivity of spectrophotometric assay is not high enough. Another advantage of the HPLC-based method is that it assesses both the formation of MSG and the depletion of MSu simultaneously, which would not be feasible for the spectrophotometric method.

Even though the GSH-conjugation of MSu was considered as GSTT2-2-specific for decades, the specificity regarding comparison with other GST isoforms has not been comprehensively investigated. In the current study, by applying the established HPLC-UV method for quantifying MSG in MSu incubations, the high specificity of MSu as a substrate for GSTT2-2 is confirmed. Even at 100 μM, the major hepatic GST isoforms GSTA1-1, GSTA2-2, and GSTM1-1 showed only a few percentage of the activity found with 50 nM GSTT2-2. Because the other GST isoforms are either way less abundant or absent in the liver, they will not contribute to MSu GSH-conjugation.

As an application of the HPLC-based method from current study, enzyme kinetics of MSu conjugation by purified recombinant GSTT2-2 and by pooled HLC were characterized. Previously, Hussey and Hayes [26] reported that purified GSTT2-2 from HLC exhibited a MSu GSH-conjugation activity of 497 nmol/min/mg protein. Later on, Tan et al. [27] described that recombinant human GSTT2-2 expressed in *E. coli* exhibited a V_{max} value of 237 nmol/min/mg protein and a K_m of 4.8 μM. These values are in the same range with maximal velocity (623 nmol/min/mg protein) and K_m (1.7 μM) obtained with the recombinant human GSTT2-2 in the present study. However, certain discrepancies were found compared with published data. For example, previous studies have never reported that enzyme kinetics of GSTT2-2 with MSu showing a substrate inhibition pattern (with the same substrate concentration range used as in the present study), whereas a clear substrate inhibition curve was observed when MSu concentrations were higher than 10 μM (Fig. 5A). Interestingly, this substrate inhibition pattern was not observed in pooled HLC, where a typical hyperbolic Michaelis-Menten curve was obtained (Fig. 5B). In addition, a 4-fold lower K_m (0.4 μM vs. 1.7 μM) was observed in incubations with pooled HLC. These differences in enzyme kinetic behaviour between HLC and recombinant GSTT2-2 may be explained by interactions of other cytosolic proteins with GSTT2-2 in case of HLC, which might have an impact on the binding of MSu to GSTT2-2. Previous studies have demonstrated that several GSTs have other functions, next to catalyzing GSH-conjugation or peroxide-hydrolysis, such as physical interaction with proteins involved in signaling pathways [28]. It therefore cannot be excluded that GSTT2-2 also has other functions.

Besides enzyme kinetic characterization of GSTT2-2, the novel HPLC-UV method was applied to characterize the variability of GSTT2-2 activity in HLCs. By comparing the activities of a panel of 20 HLC fractions, a 65-fold variation of GSTT2-2 activity was observed (Fig. 7), indicating that the inter-individual variability of this enzyme among population is very large. The basis of the large variability in this selection of HLC fractions is not known. However expression of GSTT2-2 can be determined by genetic factors, such as by mutations in the promotor region [13], and by the absence of the *GSTT2B*-pseudogenes. Furthermore, in vitro studies have demonstrated that transcription of *GSTT2* can be strongly upregulated by plant polyphenols and butyrate [29,30]. The fact that polyphenols

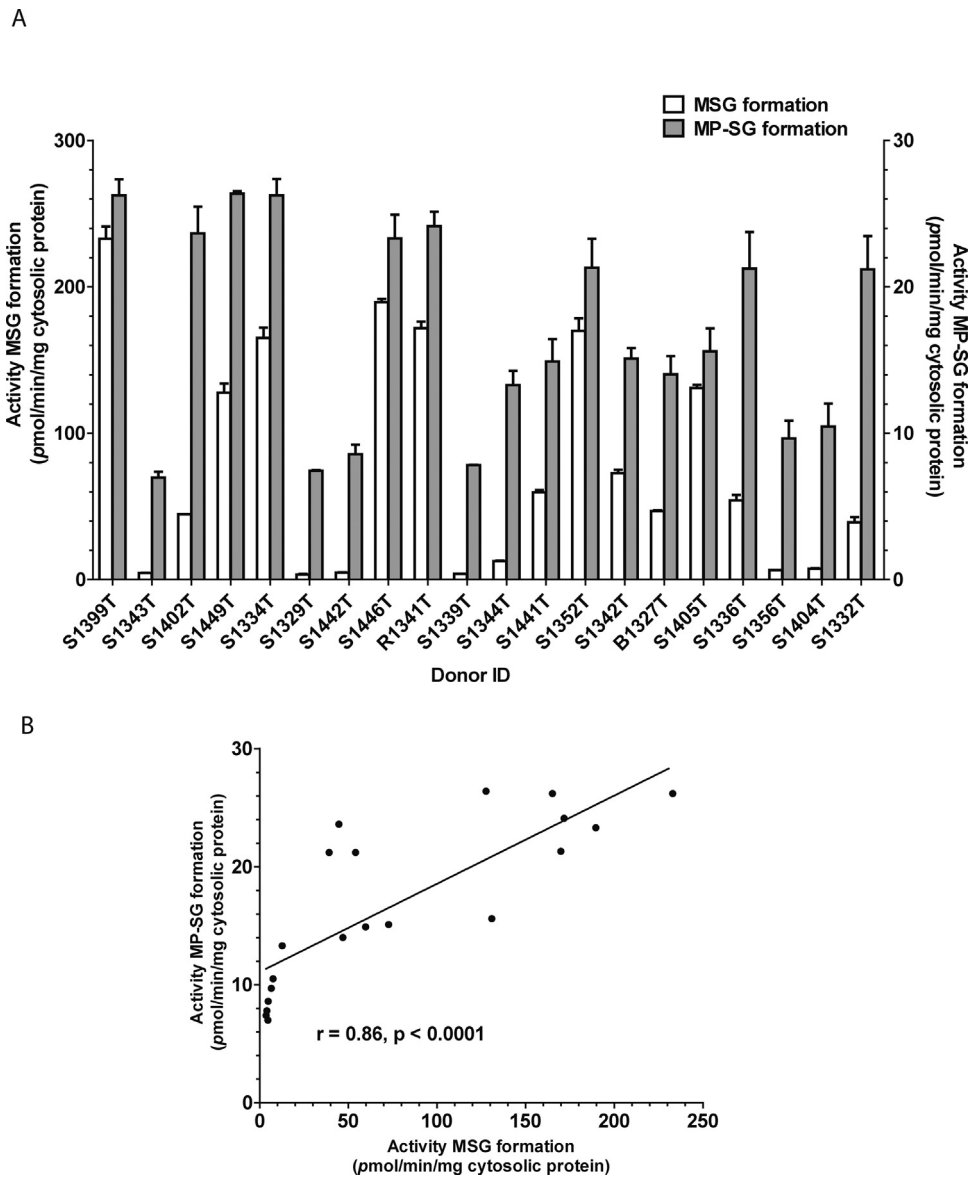

Fig. 7. Inter-individual variation of GSH-conjugation of MPS and human GSTT2-2 activity in 20 human liver donors. (A) Formation rates of MP-SG and MSG of 20 HLC. Activity of MP-SG formation is plotted as left Y-axis and activity of MSG formation is plotted as right Y-axis. Each bar represents average ± range from two separate incubations. (B) Spearman correlation analysis between GSH-conjugation of MPS and human GSTT2-2 activity (probed by MSu GSH-conjugation) in 20 human liver donors.

and butyrate provided protection against oxidative stress caused by organic peroxides is indicative that GSTT2-2 regulation is one of the important protective stress responses.

The big variability in GSTT2-2 activity will have most significant influence for (geno)toxicants which are highly dependent on GSTT2-2 for inactivation. The presence of a sulfate binding pocket in the crystal structure of GSTT2-2, suggests that the inactivation of mutagenic sulfate esters might be strongly dependent on GSTT2-2. In the present study, we examined the specificity of human GST isoforms in the detoxification of MPS. MPS is formed via sulfation of 1-hydroxylmethylpyrene (HMP), one of the major hydroxyl-metabolites of the environmental carcinogen methylpyrene [18]. The ultimate carcinogen is considered to be MPS, since this sulfate ester can spontaneously degrade to a reactive carbocation, which strongly binds to DNA. Our results show that GSTT2-2 is the most active GST catalyzing the GSH-conjugation of MPS, with the other isoforms showing less than 10% activity compared to that of GSTT2-2 (Fig. 6). However, the relatively poor correlation with GSTT2-2-activities suggests that other GSTs may also contribute to GSH-conjugation of MPS in HLC. Overall, the big variation of both GSTT2-2 activity and detoxification profiles of MPS observed in 20 individual HLC underlies a potential mechanism of inter-individual susceptibility to toxicants which are substrates of GSTT2-2.

In conclusion, in the present study a novel HPLC-based assay to quantify GSTT2-2 activity from different biological systems was validated. This new assay allows the quantification of GSTT2-2 activity in HLC, which was not feasible with previously-used method. By applying the assay, specificity of GSTT2-2 in MSu GSH-conjugation, enzyme kinetics of recombinant human GSTT2-2 and pooled HLC were characterized. More importantly, big inter-individual variability of GSTT2-2 activity and detoxification profiles of MPS were demonstrated in a small set of population, by applying this assay. Furthermore, the big inter-individual variability of GSTT2-2 activity might underlie the inter-individual differences of susceptibility to MPS carcinogenicity, the ultimate environmental carcinogen of MP.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jpba.2018.04.037.

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